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## EXECUTIVE SUMMARY

Standard toxicity tests often require high costs for maintaining healthy cultures, so few test species are employed in routine ecotoxicological analysis. An alternative is the "battery of tests" approach involving using rapid toxicity tests for screening. Rapid-screening toxicity tests must display organism sensitivity, similarity in responses to other test organisms, relevancy to many circumstances, and repeatability. Protozoa are ideal candidates for rapid-screening bioassays. They are cosmopolitan, play important roles in ecosystems, and have high reproductive rates. Many protozoa can form a resting stage (cyst) that remains viable during adverse conditions, eliminating the need for maintaining continuous cultures for testing.

The principal objective of this research was to evaluate the soil ciliate, *Colpoda inflata* (Stokes), as a bioassay organism in rapid-screening tests by determining its sensitivity to a variety of model compounds. Select experiments examined differences in response using media that affected the bioavailability of the model compounds. These tests were based on the principle that exposure to a toxic compound would negatively affect population growth. Additional studies examined the developed bioassay in a field situation and examined the potential for other, short-term sublethal indicators such as feeding rate as rapid toxicity tests. Related studies evaluated the utility of rapid-screening growth tests using the alga *Haematococcus lacustris* using methods similar to those used in the ciliate growth bioassay.

To test for sensitivity, *C. inflata* was exposed to different levels of dissolved organic carbon in test media for each compound tested. *C. inflata* was expected to be more sensitive to toxicants in an inorganic medium than in media with high organic carbon content. Data were analyzed by determining the median tolerance limit for inhibition of population growth (IG50) relative to controls. IG50 values of the eight model compounds tested varied considerably. *C. inflata* growth was not significantly affected by 2,4-D or malathion. *C. inflata* showed differences in sensitivity between organic and inorganic media for the toxic metals tested and the order of toxicity corresponded to those found in standard tests. A significant difference occurred between the test media and the pesticide PCP, where growth was not inhibited in the organic medium; in the inorganic medium the IG50 was 0.269 mg/L. No significant effect of test media was found for ammonia or SDS. Compared to several published toxicity results, *C. inflata* proved more sensitive overall than other rapid-screening tests and many standard acute toxicity tests. Results of this study show that this rapid-screening toxicity test is sensitive, repeatable, and provides information similar to traditional standard toxicity tests.

Field tests showed that the rapid test could be applied to complex mixtures in the field. Two repeat tests on samples from a low pH, contaminated groundwater gave respective IG50 values of 37% and 23% with overlapping confidence intervals.



Toxicity was removed when the pH of the contaminated water was adjusted to neutrality.

Tests of feeding rate examined the uptake of fluorescently labeled microspheres by *C. inflata* over a 20 minute period and were based on the principle that toxicants reduce feeding rate, effectively altering bioenergetic patterns and, thereby, reducing growth rate. Feeding rates were significantly reduced by copper (EC50 0.082 mg/L), a level comparable to the the IG50 for ciliate growth.

Rapid-screening tests examining the growth of *Haematococcus lacustris* showed less sensitivity than the ciliate bioassays. For the model compounds, the rank order of sensitivity was: Cu > PCP > Cd > Zn > SDS > octanol > 2,4-D. *H. lacustris* was found to be sensitive to the three metals tested, but not as sensitive as other bioassays. Also toxic was PCP, a multi-purpose biocide, acting as a cell uncoupler of oxidative phosphorylation. SDS is a surfactant and may have disrupted the cell membrane. The chlorophenoxy herbicide 2,4-D is used against broad-leafed plants. It is a growth stimulant, mimicking the plant hormone auxin and causing uncontrolled growth. Since *H. lacustris* is not a broad-leafed plant controlled by auxin the effects of this herbicide is minimal.

When ranked with eleven other bioassays, *H. lacustris* was the third most tolerant, followed by the brine shrimp and Polytox. Compared with the alga, *Selenastrum capricornutum*, *S. capricornutum* was more sensitive to most compounds tested than *H. lacustris*. However, *H. lacustris* was more sensitive to 2,4-D and was

found to be closely related in sensitivity to copper and PCP. *S. capricornutum* has a similar a rank order of sensitivity : Cd; Cu > Zn > PCP > SDS > 2,4-D to *H. lacustris*.

Microeukaryotes (protists) that produce dormant life-stages such as cysts are ideal for use in rapid-screening bioassays. The organisms can be stored in the dormant stage and grown "on demand." The experiments conducted in this project have demonstrated the simplicity and sensitivity of microeukaryote tests to be used in a "battery of tests" approach to site and contaminant screening. Bioassays based on growth (and potentially feeding rate) of the ciliate *Colpoda inflata* show sensitivity greater than most other rapid tests. Bioassays based on growth of the alga *Haematococcus lacustris*, while less sensitive, retain many of the advantages of the rapid-screening test battery. Further research will be needed to adequately characterize the breadth of compound sensitivity for the various rapid bioassays.

## 1.0

## INTRODUCTION

1.1 *Rapid screening toxicity testing*

Research in environmental toxicology has been primarily driven by legislation. There are over 63,000 chemicals in common use with 3,000 compounds accounting for approximately 90% of total usage (Maugh, 1983). Many laws require toxicity testing and toxicological assessments of compounds, effluents, discharge, or hazard waste sites. No man-made instrument can measure the toxicity of a substance to a biological organism, thus biological assays (bioassays) are necessary to estimate biological risk (Cairns, 1986; Cairns and Pratt, 1989).

Bioassays are used for regulatory purposes for several different reasons. Tebo (1985) distinguished three main purposes: screening, establishing limitations, and monitoring. At the screening level, tests should be rapid, sensitive, relevant and cost-effective. Establishing limitations requires that the tests should be precise with respect to possible contamination levels and applicable to many conditions. Monitoring tests are expected to be rapid, sensitive, precise and cost-effective.

Many different kinds of standard bioassays are currently employed. Standard test protocols include both acute and chronic toxicity, with the most common endpoint in acute toxicity being death (median lethal concentration, LC50). Standard aquatic test organisms include the green alga, *Selenastrum capricornutum*, invertebrates such as *Daphnia magna* and *Ceriodaphnia dubia* (cladocerans), and several fish species,

including *Lepomis macrochirus* (blue-gill sunfish), *Onchorhynchus mykiss* (rainbow trout), and *Pimephales promelas* (fathead minnow). This list of standard tests organisms does not represent many taxonomic groups, such as insects, bacteria, protozoa, which are abundant in the environment. Also, these tests ignore soil fauna. Organisms are chosen with the idea that they are the most sensitive indicators for their environment, which many researchers have argued is not a valid assumption (Kimball and Levin, 1985; Cairns, 1986).

Because a few single species tests may not adequately represent all environments, a "battery of tests" approach has been proposed for assessing the biological effects of contaminants in the environment (Schaeffer, 1994; Toussaint et. al, 1995). Standard tests often represent an unnecessary expenditure of resources. Thus, many alternative tests have been suggested for contaminant screening because of their simplicity, reproducibility, shorter exposure time, low cost, and increased sensitivity (Elnabarawy et. al., 1988; Snell and Persoone, 1989; Janssen and Persoone, 1993; Toussaint et. al 1995). Rapid toxicity tests are intended to be used in circumstances where screening of large numbers of samples for possible toxic effects is needed prior to definitive testing.

### 1.2 *Protozoa as test organisms*

Standard test species span a wide variety of organisms, from bacteria to fish and other vertebrates. However, the larger and more 'complex' the test organism, the more

care, cost, and time needed to maintain healthy cultures (Sprague, 1973; APHA, 1989). The test species used must be representative of the system and region tested (Landis and Yu, 1995). The common use of the cladoceran, *Daphnia magna*, to test compounds intended for the soil would not accurately depict possible effects to soil organisms. Using a cold water fish (such as a rainbow trout) to test in a region where only warm water streams occur presents another problem. In addition, special licenses are often required to conduct testing (especially with vertebrates). Therefore, bioassay organisms must be chosen using many criteria.

Protozoa are ideal candidates for environmental toxicity testing. They are small and easily cared for, saving time and resources. This is especially true when compared to organisms such as fish, for which much time and money are spent maintaining healthy stock cultures. Many protozoa form a resting stage (cyst), eliminating the necessity for constant culturing and allowing organisms to be stored until needed. The rapid growth rate of protozoa allows the effects of a compound to be assessed using an array of variables (such as reproduction, feeding, metabolism, respiration rates, etc.) in a relatively short period of time compared to other bioassay organisms (Cairns, 1974). Because of their rapid growth rate and relatively delicate nature protozoa generally react to environmental contaminants faster than other eukaryotic organisms (Foissner, 1994). In addition, most protozoa are capable of both sexual and asexual reproduction which would result in a large culture of nearly uniform genetic makeup (Cairns, 1974; Landis and Yu, 1995).

The large diversity, biomass, and production of protozoans makes them important components of both aquatic and soil ecosystems. In aquatic systems, protozoa are responsible for consumption of algae and bacteria and are essential components of the microbial loop (Fenchel, 1987). As a principal consumer of bacteria, soil protozoa (especially ciliates) help regulate and modify the size and constitution of the bacterial community (Stout, 1980). This subsequently accelerates the turnover of the soil biomass and thus the turnover of soil organic matter. Any changes in community structure and production would greatly influence higher trophic levels as well as nutrient cycling (Fenchel, 1987).

Many species of protozoa are cosmopolitan, allowing easy comparison of results from several regions and different natural systems. Protozoa may be abundant in habitats which may lack higher organisms due to extreme environmental conditions (e.g., Antarctic soils, ephemeral ponds, plant foliage, etc.). While these areas may lack higher life, man-made contaminants may exist. The eukaryotic origin of protozoa makes them comparable to metazoans, and their reactions to compounds can more convincingly be related to higher organisms than can those of the prokaryotes (Foissner, 1994). Protozoan tolerance levels are neither more nor less sensitive than those of metazoans. Lastly, special collecting/testing permits are generally not necessary.

While there are strong arguments for using protozoa as bioassay organisms, a few factors dissuade their use. Protozoa are usually inconspicuous and not detectable

to the naked eye, thus requiring the use of microscopes for identification or counting (Foissner, 1994). The large number of species often requires special training in identification. In addition, many species have not been thoroughly investigated and little or none of their ecological background is known. These factors make identification of protozoan communities a time-consuming task and supplying the needs of the culture somewhat difficult. Culturing difficulties can be mitigated, however, by using a single strain obtained through a 'library' such as the American Type Culture Collection. Responses of protozoa, like those of standard test species, cannot be directly compared to human responses or to the tolerance of an entire natural system. Finally, despite their undisputed importance in the functioning of the world's ecosystems, protozoa receive little public attention, making funding scarce.

While the use of protozoa to assess toxicity is not new, relatively few studies have been conducted. Bioassay organisms include *Tetrahymena pyriformis* (e.g., Yoshioka, et al., 1985; APHA, 1989; Roberts and Berk, 1993), *Tetrahymena thermophila* (Pauli et al., 1993), *Entosiphon sulcatum* (Bringmann and Kuhn, 1980), *Spirostomum ambiguum* (Nalecz-Jawecki et al., 1993), *Colpoda cucculus* (Janssen et al., 1995), *Colpoda stenii* (Forge et al., 1993) and *Colpidium campylum* (e.g., Dive et al., 1980; Dive et al., 1982; Dive et al., 1989; Le Du et al., 1993). Methods used for protozoan bioassays differ considerably. Endpoints measured include lethality, (Ruthven and Cairns, 1973), chemoattraction (Roberts and Berk, 1993) and deformation (Nalecz-Jawecki et al., 1993). The most common method is the

assessment of growth inhibition based on population density. Several of these tests, including all tests conducted with *C. campylum*, were based on counting populations by means of an electronic cell counter (e.g., a Coulter Electronic Particle Counter; Gray and Ventilla, 1973; Dive et al., 1989). Others, such as the *T. pyriformis* (the only protozoan test in *Standard Methods*, APHA, 1989) used change in absorbance measured with a spectrophotometer) as an indication of effects of compounds. Counting has also been performed manually by microscopy (Forge et al., 1993; Janssen et al., 1995). Protozoan bioassays have been used to test the effects of many compounds including: pesticides (Dive et al., 1980; Schreiber and Brink, 1989), metals (Rutheven and Cairns, 1973; Dive et al., 1982), oils and detergents (Rogerson and Berger, 1981) and a variety of compounds and mixtures (e.g., sewage sludge and effluent). Because methods were sometimes drastically different, few generalizations can be made concerning the sensitivity of individual species or tests. However, it appears that protozoan bioassays display varying responses to different chemicals within and between species (Rutheven and Cairns, 1973; Dive et al., 1980; Nalecz-Jawecki et al., 1993).

### 1.3 *Colpoda inflata*

Relatively little environmental research has been conducted on *Colpoda inflata* (Stokes), (Protozoa: Ciliophora), the ciliate protozoan used in this research. It appears to be cosmopolitan, its presence having been documented in many countries (Foissner,



1993). Genetic analysis of strains reveal no significant differences among *C. inflata* from fifteen different locations spanning biogeographic regions (Bowers and Pratt, 1995). Cells range in size from 30 -90 um, but always display a characteristic L- shape (Figure 1). *C. inflata* feeds almost entirely on bacteria, but has also been observed to consume small flagellates (Foissner, 1993; Foissner and Berger, 1996). While *C. inflata* can inhabit both freshwater and soil, it is more commonly associated with terrestrial habitats and is generally accepted to be a soil protozoan (Foissner, 1993). Consequently, it displays features common to other terrestrial protozoa.

Soil protozoa must be able to adapt to the flux of moisture in their environment. Loss of water due to evaporation and evapotranspiration increases the relative concentration of salts, producing osmotic stress. Heavy or extended precipitation may cause dilution stress and create anaerobic conditions by filling available airspaces between soil particles (Fenchel, 1987). A survival trait of most soil protozoa is the ability to produce resting stages, which allow the cell to remain viable within temporary sheath. Resistant cysts are formed when environmental conditions become unfavorable (e.g., decreased availability of a food source, reduced oxygen, desiccation, change in pH, etc.). When environmental conditions improve, soil protozoa will excyst (i.e., return to active form). The time of excystment is variable, but some research has shown that there is an inverse relationship between the amount of time a soil protozoan remains in a resting cyst and the amount of time it will take to excyst (Loussier and

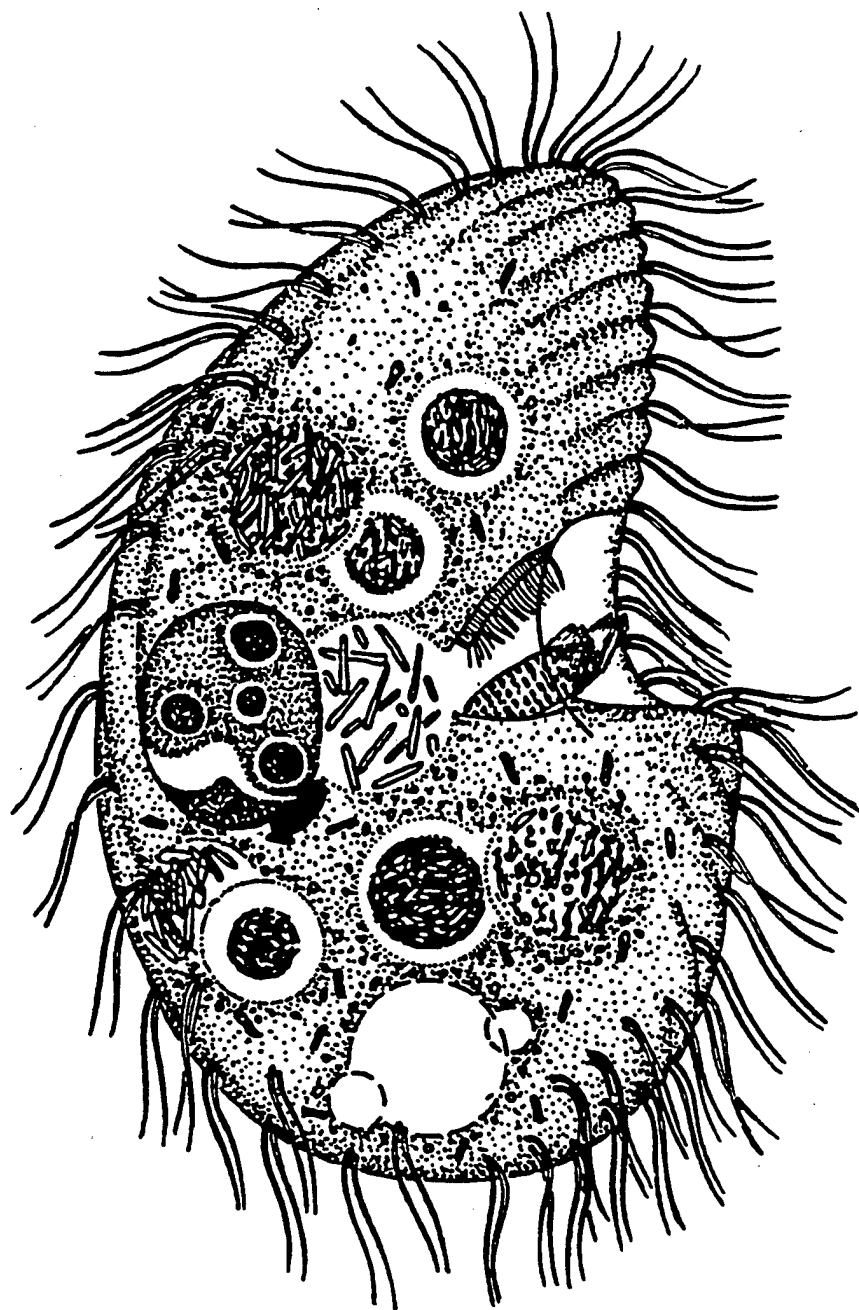


Figure 1.1. *Colpoda inflata* (from Foissner, 1993). The typically “L-shaped” cells are 40-60  $\mu\text{m}$  in length.

Bamforth, 1990). Initial studies with *C. inflata* have shown that excystment rate is extremely variable (Zhenkang Xu, pers. comm).

Sexual reproduction has never been documented in *C. inflata* (Foissner, 1993). Instead, these ciliates reproduce asexually by binary fission. In *C. inflata*, two consecutive divisions occur within special reproductive cysts (which generally have thinner walls than resting cysts). These divisions are transverse (i.e., occur at right angles) and produce 4 viable cells. Studies have found that the generation is 6.2 hours time at 15 °C and 2.6 hours at 30°C (Foissner, 1993).

#### 1.4 Bioavailability

Organic substances in the environment often influence the solubility, mobility, persistence and toxicity of contaminants. Natural organic substances are derived from decomposition of dead organisms, animal wastes, and microbial byproducts. The term bioavailable refers to the proportion of a compound in the environment that may be taken up by an organism (Cockerham and Shane, 1994). Compounds may bind to dissolved complexes, sediments, or organic films, therefore potentially decreasing their availability to organisms and, therefore, their toxicity. However, there is a lack of quantitative data to explain this phenomenon and unfortunately almost no predictive capability currently exists to forecast bioavailability without large uncertainty (Depledge et al., 1994). Sensitive and biologically accurate models are desperately

needed to discriminate toxicity caused by binding of organic material (Jenne and Zachara, 1987).

Protozoa are often found in environments that contain a large amount of organic material (e.g., dissolved organic carbon (DOC)). Natural concentrations of DOC in streams and rivers can be as high as 50 mg carbon/L in waters (Stumm and Morgan, 1996). DOC has been shown to alter toxicity of several metals (Depledge et al., 1994) as well as decomposition rates of certain pesticides (Perdue and Wolfe, 1982). Most protozoan culture media also contains high amounts of DOC, usually to grow the bacterial food source. Inorganic media would require the constant addition of a food supply (i.e., bacteria), since few protozoa can be cultured axenically. While higher organisms are often tested and cultured in "natural" media (i.e., well water, reconstituted water), to test for bioavailability would take a significantly longer time than it would for a protozoan bioassay. Dive et. al. (1982) found the ciliate, *Colpidium campylum*, can be used to detect cadmium complexation to humic material as a measured by differential population growth. They found that both toxicity and complexation of cadmium are highly correlated to medium composition. Yoshioka et al. (1985) suggested that the composition of the test medium used to grow *Tetrahymena pyriformis* could alter the toxicity of a given compound. Therefore the use of ciliates to evaluate bioavailability of compounds is promising.

### 1.5 *Supplemental Studies*

Field studies of contaminated ground and surface water were conducted at the Edgewood Army Arsenal in Edgewood, MD using the *C. inflata* growth test.

Preliminary tests were also conducted using ingestion rates of the ciliate, *C. inflata* as a toxicity endpoint. Growth measurements (i.e. energy needed for individual growth) are considered to be a useful appraisal of organism stress to a toxicant. Because growth is related to birth and death rates, it can affect population composition. It is possible to estimate the energy available for growth (the scope of growth, SFG) by measuring rates of ingestion, respiration, excretion and metabolism. Theoretically, growth would show a response when any of the factors influencing growth are disturbed, so any changes in bioenergetics of individual would affect SFG and therefore indicate a toxicological response. (Forbes and Forbes, 1994). According to Atchison et al. (1996), when food levels are constant, toxicants can reduce the filtration rate and the "apparent food concentration" below levels required for reproduction and population maintenance.

### 1.6 *Haematococcus lacustris*

Algae are ubiquitous and important organisms serving as primary producers in both aquatic and terrestrial systems. Since they are the base of most food chains, bioassays using algae are vital to the understanding of the effects of contaminants to an

ecosystem. There are currently several bioassays which use algae as test organisms. These usually measure population growth by investigating change in biomass. This can be accomplished by analysis of chlorophyll a, respiration, dry mass or cell numbers (APHA, 1989). The most widely used standard test alga is *Selenastrum capricornutum*.

This portion of the research investigated the use of *Haematococcus lacustris* (Fig. 1.2) as a bioassay organism by using rapid methods similar to those used for *Colpoda inflata* bioassays. *H. lacustris* is a unicellular alga ranging in size from 10 to 50um in diameter. Cells have a wide sheath-like wall (the protoplast) connected by fine fibrils to the wall margins. Active cells are ovoid in shape and possess two flagella. Under adverse environmental conditions, such as intense illumination, lack of water, nutrients, light, *H. lacustris* forms a resting cyst in which the green chloroplast is obscured by orange-red carotenoid (haematochrome). This characteristic allows the active and cyst forms to be easily distinguished (Prescott, 1982).

*H. lacustris* can often be found in small pools, crevices holding rain water, and cement basins worldwide. A garden bird-bath is a common habitat for *H. lacustris*, where because of frequent periods of drying it most likely appears as a brick-red mass (Prescott, 1982).

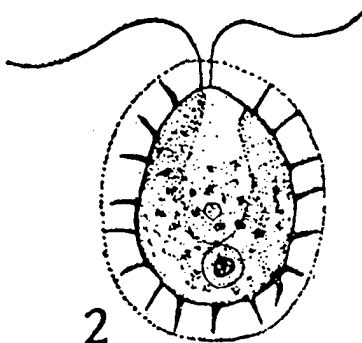


Figure 1.2 *Haematococcus lacustris* (from Prescott, 1951). Flagellated algal cells typically with an enlarged cell envelope and reddish color due to the presence of the pigment hematochrome.

### 1.7 Objectives

The objectives of the present study were as follows.

- 1) Investigate the use of the ciliated protozoan, *Colpoda inflata*, as a bioassay organism by determining its sensitivity to model compounds and complex mixtures.
- 2) Assess effects of media on bioavailability by using organic and inorganic media to compare sensitivity to model compounds.
- 3) Evaluate the effectiveness of *Colpoda inflata* ingestion rate as an endpoint to indicate toxicity.
- 4) Investigate the use of the alga, *Haematococcus lacustris*, as a bioassay organism by determining its sensitivity to model contaminants.

Test methods emphasized simplicity in order to fulfill the definition of rapid-screening tests and to also allow practical use as a semi-field toxicity test in the future.

## 2.0

## MATERIALS AND METHODS

2.1 *Colpoda inflata* growth test*Test Principle*

These toxicity tests are based on the principle that toxicity of a compound will negatively affect population growth of the soil ciliate, *C. inflata*. Estimates of toxicity using growth rates are based on growth in controls. Normal growth rates are relatively high (3-4 doublings per day) and the cells are large enough to be counted accurately under low magnification (40-80x). A test involves inoculating medium and toxicant to a fixed number of cells and counting cells after 24 hours. Toxicity is then inferred by difference in cell number across doses (i.e., difference in growth rate). These tests are an ideal candidate for a rapid screening of toxicants.

*Test organism*

The strain of *C. inflata* used in this research was obtained as dry cysts from the American Type Culture Collection (ATCC 30917, Rockville, MD, USA). A non-pathogenic strain of the bacterium *Klebsiella pneumoniae* (ATCC 27889) was used as a food source in the inorganic media.



### *Test compounds*

Media preparation. To assess the effects of media type on various compounds, two media classes (inorganic and organic) were used in the *C. inflata* experiments. The inorganic minimal salt medium (6 mg KCL, 4 mg CaHPO<sub>4</sub>, 2 mg MgSO<sub>4</sub>, 1 L deionized distilled water (ddH<sub>2</sub>O); Prescott and James, 1955) was sterilized before each use. The organic medium, Sonneborn's *Paramecium* Medium, was prepared by boiling 2.5 g Cerophyll for 5 minutes in 1 L ddH<sub>2</sub>O, filtered through #1 Whatman filter paper buffered with 0.5 g Na<sub>2</sub>PO<sub>4</sub>, and autoclaved. Full strength Sonneborn's was diluted to a final concentration of 10% and 5%. All compounds were tested using both minimal salts medium and 10% Sonneborn's. Final averaged pHs of minimal salts and 10% Sonneborn's were 5.5 and 6.8, respectively. Three compounds (the metals) were also tested with 5% Sonneborn's.

Stock preparation. Eleven compounds were tested: ammonia (as (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>), cadmium (as 3CdSO<sub>4</sub> · 2H<sub>2</sub>O), copper (as CuSO<sub>4</sub> · 5H<sub>2</sub>O), 2,4-dichlorophenoxyacetic acid (2,4-D), malathion, octanol, pentachlorophenol (PCP), phenol, sodium dodecyl sulfate (SDS as laural sulfate), 2,4,6- trinitrotoluene (TNT) and zinc (as ZnSO<sub>4</sub> · 7H<sub>2</sub>O) (Table 1). Chemical sources included: J.T. Baker Inc. (ammonium sulfate, cadmium, copper), Cheminova, Inc. (malathion), Mallinckrodt Chemical Works (zinc), Rhone-Poulenc, Inc. (2,4-D), Sigma Chemical Company (PCP, phenol, SDS) and the Army Biomedical Research and Development Laboratory in Ft. Detrick, Maryland (Octanol, TNT). Cadmium, copper, and zinc were prepared with ddH<sub>2</sub>O; malathion, PCP,

phenol, 2,4-D, ammonia and SDS were prepared using both sterilized minimal salts and 10% Sonneborn's media. Octanol and TNT were prepared using sterilized minimal salts media. Prepared stock concentrations and analyzes are listed in Table 1. Octanol and TNT was prepared by the Army Biomedical Research and Development Laboratory, Ft. Detrick, Maryland. Final stocks of malathion, PCP, un-ionized ammonia, and SDS did not need pH adjustments. The pH of the 2,4-D stock made with minimal salts medium was adjusted with 0.15 NaOH. No carrier solvents were used.

Table 2.1. Prepared and measured stock concentrations (in mg/l) of nine compounds. Analytical methods of analysis chosen by Coffey Laboratories, Inc.

Compound	Prepared concentration	Actual concentration	Analytical method
Ammonia (as $(\text{NH}_4)_2 \text{SO}_4$ )	1000	670 un-ionized= 0.0944	SM 4500, A-D
Cadmium (as $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ )	1	0.72	CFR 200.7
	5	6.5	
Copper (as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ )	5	5.4	CFR 200.7
	50	51	
2,4- dichlorophenoxyacetic acid (2,4-D)	600	280	EPA 615
Malathion	140	120	EPA 614
Pentachlorophenol (PCP)	14	2	CFR 604
Phenol	94	94	CFR 604
Sodium Dodecyl Sulfate (SDS) (as laural sulfate)	1000	1100	CFR 200.7
Zinc (as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ )	10	11	CFR 200.7
	100	110	

Toxicant verification and waste disposal. For each compound tested, toxicant concentration of primary and secondary stocks was independently verified by Coffey Laboratories, Inc. (a certified analytical laboratory, Portland, OR). Octanol and TNT were prepared and verified by the Biomedical Research and Development Laboratory, Ft. Detrick, Maryland. Table 2.1 lists the estimated stock concentrations and actual stock concentrations based on methods listed. Chemical preservation procedures followed the recommendations of Coffey Laboratories, and were based on the specific compound used. Analytical methods employed are briefly described in appendix B. Waste disposal followed normal Portland State University procedures (disposal through Environmental Health and Safety).

### *Test Methods*

Culturing. The food bacterium, *K. pneumoniae*, was cultured on Bacto Nutrient Agar plates (APHA, 1989) at room temperature. After adequate growth (less than a week), the bacteria were scraped from the plates and placed in microcentrifuge tubes. These tubes were then placed in the freezer (-20° C) until needed. The day of the test, the tube of *K. pneumoniae* was taken out of the freezer and warmed at room temperature. A small quantity (approximately 10 mg) of bacteria was taken from the tube, placed in a small plastic beaker and weighed. Approximately 20 ml of minimal salts medium was added to the beaker, which was subsequently covered with parafilm and vigorously vortexed. After adequate mixing, minimal salts media was added

slowly to the bacterial solution and optical densities are taken after each addition. Previous experimentation determined ample bacteria for ciliate growth was at an absorbance of approximately 0.2 at 400 nm. At this absorbance, the bacterial density is approximately  $3.2 \times 10^7$  cells per ml. (Appendix A).

To begin a culture, the *C. inflata* cysts were inoculated in sterile 12-well polystyrene tissue culture plates (Costar, Inc.) with 2.5 ml 10 % Sonneborn's medium and incubated at 25 °C. Once cysts hatch, culture wells were subsampled daily to keep population in exponential growth and also to remove waste and add new medium.

Cell enumeration. Subsamples of wells with large populations were removed with a disposable transfer pipette and placed into 20-ml scintillation vials. The culture was then mixed and 20 µl withdrawn with an automatic micropipettor and dispensed via 4-6 drops on a clean microscope slide. Using a stereo microscope (magnification 40-80x) the total number of *C. inflata* cells in 20 µl were estimated by counting each drop. This 20 µl count was repeated at least three times. If counts varied by more than 20%, improper mixing may have occurred and the process was repeated. When the average number of cells per 20 µl was determined, the number of cells per ml (1000 µl) of culture was calculated by multiplying the average 20 µl count by 50.

Initiating a test. A single test consisted of 4 replicates of 6 treatments for a total of 24 individual experimental units. Treatment concentrations were arranged logarithmically and the volumes of culture, medium, and toxicant were determined before each experiment (total well volume is 2 ml). Stock culture density was

determined (method described previously) so that a constant number of cells (approximately 200) were placed in each well. Once the culture volume was determined (culture volume never exceeded 200  $\mu$ l), volumes of media and toxicants were calculated. For tests with the inorganic media, a constant volume of bacteria (200  $\mu$ l) was added to every well in test performed. Medium and bacteria for the minimal salts medium were carefully added to each well of a 24-well polystyrene tissue culture plate. The previously determined aliquots of well mixed culture were distributed next. The toxicant was added last, at which point the test began. After test initiation, the plate was incubated at 25 °C for 24 hours ( $\pm$  2 hr). At the end of the test period, the density of cells in each replicate well was estimated by cell enumeration.

Data analysis. Data were analyzed in several ways. The endpoint examined was the density of *C. inflata* cells at the end of a test period. Cell densities were compared using a single factor analysis of variance test (6 treatments, 4 replicates per treatment). Dunnett's test was used to determine responses which were significantly different from the control (Dunnett, 1955).

Data also were examined by identifying the median tolerance limit for population growth, defined as the concentration corresponding to a 50% inhibition of growth (IG50) relative to controls. This was accomplished by linear regression and inverse prediction (Sokal and Rohlf, 1995), estimating the IG50 from the control response where control mean was 100%. Only the data from linear section of the dose-response relationship were analyzed (i.e treatments higher than the lowest

concentration of zero growth were discarded). Estimates of final treatment concentrations were extrapolated based on measured stock concentrations given by Coffey Laboratories, Inc. IG50s were considered statistically different if their 95% confidence intervals did not overlap.

## 2.2 Supplemental studies

Field testing. Field tests of complex mixtures were conducted at the Edgewood Army Arsenal in Edgewood, MD. All tests were performed in the Biomedical Research and Development Laboratory's mobile aquatic toxicology trailer. Test methods were similar to those described previously. Three sites were tested, one stream (West Branch Canal Creek) and one groundwater site (surface aquifer at West Branch Canal Creek). Water taken from the West Branch Canal Creek was filtered because invertebrates (other protozoans, rotifers, etc) were present. The groundwater sample had a pH of 4.1. Groundwater tests were conducted with pH 4.1 and also with the pH adjusted to 7.1.

Ingestion rate. This test is based on the principle that the ingestion rate of *C. inflata* will be negatively affected by an increase in toxicity. In this instance, ingestion rate was estimated by introducing fluorescently labeled microspheres (FLM) at tracer levels to *C. inflata* exposed to a toxicant. Ingestion rate was then estimated based on comparison to the controls. *C. inflata* is a filter feeder capable of filtering particles of a variety of effective diameters. Previous studies in our laboratory have indicated that

particles of diameter 0.5 - 4.0  $\mu\text{m}$  are retained on the filter and that there are not significant differences between filtration of FLM and fluorescently stained food bacteria (Z. Xu, unpublished). This corresponds to other reports of optimal particle size for small-mouthed ciliates like *Colpoda*, *Colpidium*, and *Glaucoma* (Fenchel, 1987).

Organisms were cultured and enumerated as described above (see section 2.1). Tests were conducted using minimal salts medium (see section 2.1) in 24-well polystyrene tissue culture plate (Costar, Inc.). A single test consisted of seven treatments with three replicates. To start a test, volumes of toxicant and minimal salts medium were calculated based on: *C. inflata* density (150 cells/ml; no more than 200  $\mu\text{l}$  added), 0.5  $\mu\text{m}$  diameter FLM density (43.5  $\mu\text{l}$  is  $10^6$  beads/ml), and bacteria (200  $\mu\text{l}$ ). Each test well consisted of a total volume of 2 ml.

On the day of the test, the bacteria, *Klebsiella pneumoniae*, was taken from the freezer and prepared as in section 2.1. Minimal salts medium was added to each well, followed by previously determined volumes of *C. inflata*, *K. pneumoniae*, and toxicant. *C. inflata* was allowed to feed for one hour before the addition of FLM. Twenty minutes later, each well was fixed with 10% glutaraldehyde (GTA) and the entire contents were transferred to microcentrifuge tubes. The tubes were placed in the refrigerator (4° C) for 24 hrs to permit settling. A 6  $\mu\text{l}$  subsample was taken from each tube. The number of FLM ingested per *C. inflata* were counted at 1000X under fluorescent light. Fifteen *C. inflata* were counted for each test well.

Previous experimentation indicated that there was no significant difference between test wells. The endpoint examined was the ingestion rate (i.e. number of FLM/ *C. inflata*) at the end of the test period. Ingestion rate was compared using a single factor analysis of variance (6 treatments, 3 replicates per treatment). Dunnett's test was used to determine significantly different responses (Dunnett, 1955). Data were also examined using linear regression and inverse prediction to predict effective concentrations for feeding inhibition (Sokal and Rohlf, 1995).

### 2.3 *Haematococcus lacustris*

#### *Test Principle*

These tests are based on the principle that the toxicity of a compound will negatively affect population growth of *H. lacustris*. Estimates of population growth rate are based on comparisons to control growth.

#### *Test Organism*

The strain of *H. lacustris* was obtained as dry cysts from the American Type Culture Collection (ATCC 30402, Rockville, MD, USA).

Culture medium. Algae were cultured and tested in modified Bristol's Solution. This medium is a minimal salts growth medium containing  $\text{NaNO}_3$ ,  $\text{CaCl}_2$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4$  and  $\text{NaCl}$ , with a drop of 1%  $\text{FeCl}_3$  solution



(ATCC culture medium 847). The pH of the final media was 7. Medium was sterilized before use.

### *Test compounds*

Stock preparation. Seven compounds were tested: cadmium, copper, 2,4-dichlorophenoxyacetic acid (2,4-D), octanol, pentachlorophenol (PCP), sodium dodecyl sulfate (SDS) and zinc. All compounds were prepared as stock solutions in distilled deionized water. Metals were prepared from sulfate salts. No carrier solvents were used.

### *Test Methods*

Culturing. *H. lacustris* was cultured in 75 mL Erlenmeyer flasks with Bristol's medium under a constant fluorescent (cool white) light source.

Cell enumeration. The stock culture was mixed, and 20  $\mu$ l was withdrawn with an automatic micropipettor and dispensed via 4-6 drops on a clean microscope slide. Using a stereo microscope (magnification 40-80x) the total number of *H. lacustris* cells in 20  $\mu$ l was estimated by counting all cells in each drop. This 20  $\mu$ l count was repeated at least three times. If counts varied by more than 20%, improper mixing may have occurred and the process was repeated. When the average number of cells per 20  $\mu$ l was determined, the number of cells per ml (1000  $\mu$ l) of culture was calculated by multiplying the average 20  $\mu$ l count by 50.

Initiating a test. One test consisted of at least 6 treatments (control and five test concentrations) with four replicates each. Treatment concentrations were arranged logarithmically and the volumes of culture, medium, and toxicant were determined before each experiment. Stock culture density was determined (see method described previously for *C. inflata*) so that a constant number of cells (approximately 30,000) were placed in each well of a 24 well polystyrene tissue culture plate (Costar, Inc.). Once the culture volume was determined, volumes of media and toxicants were calculated (total well volume was 2ml). The toxicant was added last, at which point the test began. After test initiation, the plate was placed approximately 27 cm under continuous fluorescent (cool white) light for 24 hours ( $\pm 2$  hr) at 20° C. At the end of the test period, the density of cells in each replicate well was estimated by direct counting as described above.

Data analysis. Data were analyzed in several ways. The endpoint examined was the density of *H. lacustris* cells at the end of a test period. Cell densities were compared using a single factor analysis of variance test (6 treatments, 4 replicates per treatment). Dunnett's test was used to determine responses which were significantly different from the control (Dunnett, 1955).

Data also were examined by identifying the median tolerance limit for population growth, defined as the concentration corresponding to a 50% inhibition of growth (IG50) relative to controls. This was accomplished by linear regression and inverse prediction without controls (Sokal and Rohlf, 1995), estimating the IG50 from

the control response where the control mean was 100%. Only the data from linear section of the dose-response relationship were analyzed (i.e., treatments higher than the lowest concentration of zero growth were discarded). IG50s were considered statistically different if their 95% confidence intervals did not overlap.

## 3.0

## RESULTS AND DISCUSSION

3.1 *Colpoda inflata* growth test

Each compound was tested in duplicate with a total of 48 test wells (40 dosed, 8 control wells). Tests of a single compound were conducted on the same day, thus using the same protozoa and bacteria cultures and stocks of both the compound and the media.

*Sensitivity*

*C. inflata* displayed varying sensitivity to the eleven compounds tested (Table 3.1). Values calculated for IG50s ranged from 0.020 mg/L to > 231 mg/L. Of the eleven compounds evaluated, two pesticides (2,4-D and malathion) and phenol did not significantly inhibit *C. inflata* growth at the highest concentrations tested. The rank order of sensitivity in the minimal salts medium was: un-ionized ammonia>Cd>Cu>Zn>PCP>TNT>octanol>SDS>phenol; 2,4-D; malathion. IG50 values for the majority of the toxicants were on the lower end of the acute toxicity range. This acute range is based on toxicity from several invertebrates (crustaceans and insects) and fish (USEPA, 1986). However, IG50s were higher than the water quality range indicating that at this level of protection, *C. inflata* population growth should not be significantly affected.

Table 3.1. Sensitivity (expressed as inhibition of growth for 50% of the population, IG50, no observed effect concentration, NOEC, and lowest observed effect concentration, LOEC) of *C. inflata* to 11 compounds in 24 hr tests. Values in parentheses are 95% confidence intervals. Tests were conducted in minimal salts media. Acute toxicity range is based on water quality criteria (USEPA, 1986). All values are mg/L. Dashes (--) identify data for which values could not be determined or values for which criteria are not available.

Compound	IG50	LOEC	NOEC	Acute Toxicity Range	Water Quality Criteria Range
Ammonia (un-ionized)	0.019 (0.013-0.029)	0.0094	--	0.083-23 (uncorrected)	0.0019-0.051
Cd	0.020 (0.01-0.03)	0.01	0.0072	0.001-28	0.0011-0.0039
Cu	0.059 (0.02-0.14)	0.06	--	0.017-10	0.012-0.018
2,4-D	> 230	--	230	37-130	0.1
Malathion	> 99	--	99	0.00076-0.29	0.0001
Octanol	14 (8.5-25)	12.9	4.8	--	--
PCP	0.27 (0.18-0.39)	0.28	0.14	0.0044-44	0.013-0.020
Phenol	> 80	--	80	10	2
SDS	52 (38-74)	33	20	15	--
TNT	9.23 (2.2-25)	2	1	--	--
Zn	0.13 (0.08-0.21)	--	--	0.051-89	0.11-0.12

Table 3.2. EC50/LC50 values of several species of protozoa (in mg/L). Ranges indicate that EC50/LC50 values were found more than once for the same test species. Dashes (--) indicate comparisons for which data were unavailable.

Test Organism	Ammonia	Cd	Cu	2,4-D	Malathion	PCP	SDS	Zn
<i>Colpidium campyllum</i> <sup>a</sup>	--	0.05-0.171	0.17	> 10	>10	0.6-1.60	--	0.80
<i>Colpoda cucullus</i> <sup>b</sup>	--	0.22	0.97	--	--	--	--	1.37
<i>Colpoda inflata</i> <sup>c</sup>	0.019	0.02	0.059	>230	>99	0.27	53	0.13
<i>Colpoda stenii</i> <sup>d</sup>	--	0.22	0.25	--	--	--	--	0.85
<i>Entosiphon sulcatum</i> <sup>e</sup>	--	0.011	0.11	--	--	--	40	--
<i>Spirostomum ambiguum</i> <sup>f</sup>	--	0.078-5.27	0.004-0.006	--	--	--	1.63-12.1	--
<i>Tetrahymena pyriformis</i> <sup>g</sup>	--	0.038-0.112	0.045-0.132	> 500	--	0.15-0.30	--	--

a: Le Du et. al, 1993; Dive et al., 1989; Dive et. al., 1980 (24 hr EC50, organic medium); b: Janssen et. al, 1995 (7 d EC50, organic medium); c: This study (24 hr IG50/EC50, inorganic medium); d: Forge et. al, 1993 (24 hr EC50, inorganic medium); e: Bringmann and Kuhn, 1980 (72 hr EC50, hard water); f: Nalecz-Jawecki et al., 1993 (24 hr LC50, hard and soft water); g: Roberts and Berk, 1993 (1hr, 5hr EC50, 24 hr LC50, organic medium); Yoshioka et al., 1985 (24 hr EC50, organic medium)

Table 3.2 lists the median effective concentration (EC50) or the median lethal concentration (LC50) of several species of protozoa to eight compounds studied in this research. Many of these experiments were conducted under substantially different test conditions, so direct comparison would be unreasonable. However, it is a good representative account of current protozoan toxicology. Cadmium and copper were the most studied with these species, followed by zinc. A few studies have also been performed with PCP, SDS, and 2,4-D, but, only one other investigation involved malathion and phenol. No documentation on toxicity of ammonia, octanol, nor TNT to protozoa was found.

According to the table, considerable differences in responses to compounds exist among and within species. Variation among species could be due to differences in test conditions, but it is interesting to note that there are some similarities. Values for copper were never higher than 0.97 mg/L. For cadmium and zinc, only one value was higher than 1.0 mg/L and that was most likely an artifact due to methodology. The 5.27 mg/L LC50 for cadmium on *S. ambiguum* was tested in hard water and the EC50 of zinc on *C. cucullus* (1.37 mg/L) was calculated over a seven day period. All tests with 2,4-D and malathion found no effect. All tests conducted with PCP revealed toxicity values below 1.6 mg/L. The PCP EC50 values (0.6 mg/L and 1.6 mg/L) with *C. campylum* were determined with the same methods. SDS values ranged from 1.63 mg/L to 52.24 mg/L. Large differences to tested compounds also occurred within each

species, even when the same test method was used. Therefore, like *C. inflata*, other protozoa seem to be sensitive to some compounds and more tolerant of others.

A comparison of several rapid-screening toxicity tests with standard toxicity tests is listed in Table 3.3. There was significant variation in responses among test organisms and it should be noted that no species was the most sensitive to all compounds. When compared with all tests, *C. inflata* was the most sensitive to cadmium and un-ionized ammonia. For copper, the rotifer, *Branchionus calyciflorus*, and *Ceriodaphnia dubia* were the most sensitive followed by *Daphnia magna* and *Selenastrum capricornutum*. *C. inflata* displayed sensitivities close to the standard tests of *D. magna* and *S. capricornutum*. The brine shrimp and Polytox® (a bacteria mixture) were similar to *C. inflata* in the tolerance to 2,4-D and malathion. The lettuce root growth test and *D. magna* were by far the most sensitive to 2,4-D and malathion, respectively. *C. inflata* ranked among the most sensitive organisms for PCP (the fathead minnow, *Pimephales promelas*, *C. dubia* and *D. magna*) and for zinc (*C. dubia* and *S. capricornutum*). The IG50 for SDS is comparable to the LC50 for *C. dubia*, which is the second most tolerant to this compound. Both IG50s for octanol and TNT displayed moderate tolerance levels when compared to the other tests.

A ranking of mean sensitivity to eleven compounds by toxicity was calculated and is shown in Figure 3.1. The most sensitive test for each compound was scored as a one, and the number increased as tolerance increased (most tolerant scored as 11). Values which were not found or were not determined (greater than EC50/LC50s) were



not ranked. For each test organism, the ranks were summed and averaged to estimate the average sensitivity. The order of sensitivity of test organisms was: *S.*

*capricornutum* > *M. bahia* > *C. inflata* > Lettuce > *C. dubia* > *B. calyciflorus* (rotifer) > *P. promelas* > Microtox® > *D. magna* > *H. lacustris* > brine shrimp > Polytox®. *C. inflata* appears to be one of the most sensitive test organisms on this list.

Previous experiments with metal compounds, conducted by several undergraduates, yielded similar IG50s to those in this study. Test methods were slightly different, but comparable in the use of 10% Sonneborn's medium (J.R. Pratt, unpublished research). Also, preliminary tests conducted for this research showed low variability among tests conducted on separate days. These data are not included because they occurred before a standard method was determined. Given this information, it can be concluded that this test gives repeatable results when conducted by different technicians as well as with different cultures, stocks, and test media.

Table 3.3. LC50/EC50 endpoints (in mg/L) of several rapid and standard toxicity tests currently being utilized (from Toussaint et. al., 1995). Dashes (--) identify comparisons for which data were not available.

Compound	Rapid Toxicity Tests					Standard Toxicity Tests					
	<i>C. inflata</i>	Rotifer	Microtox®	Brine Shrimp	Polytox®	Lettuce	<i>D. magna</i>	<i>C. dubia</i>	<i>S. capricornutum</i>	<i>P. promelas</i>	<i>M. bahia</i>
Ammonia	0.019	4.6	1.75	14.6	>20	0.03	1.5	2.18	--	1.59	1.4
Cadmium	0.020	1.3	102	160	46	0.06	0.065	0.11	0.04	2.02	0.03
Copper	0.059	0.026	1.3	4.9	38	0.62	0.052	0.027	0.04	0.48	0.16
2,4-D	> 230	117	101	>151	>302	<0.5	25	236	95.8	227	--
Malathion	> 99	33.7	10	>25	>50	>53	0.001	--	--	11.8	4.2
Octanol	14	> 96	6.12	58.9	126	11.9	36.6	8.7	--	13.5	--
PCP	0.27	1.2	1.02	0.51	90	0.59	0.37	0.28	0.34	0.23	--
Phenol	> 80	> 150	26	157	1,410	51.7	13.2	4.3	61.1	30.3	--
SDS	53	1.4	1.8	19.1	470	5.34	9.6	48.4	3.75	8.0	6.6
TNT	9.2	5.55	10.8	29.1	> 100	2.34	11.7	--	2.0	3.1	--
Zinc	0.13	1.3	12	>20	31	2.44	0.56	0.076	0.06	2.65	0.499

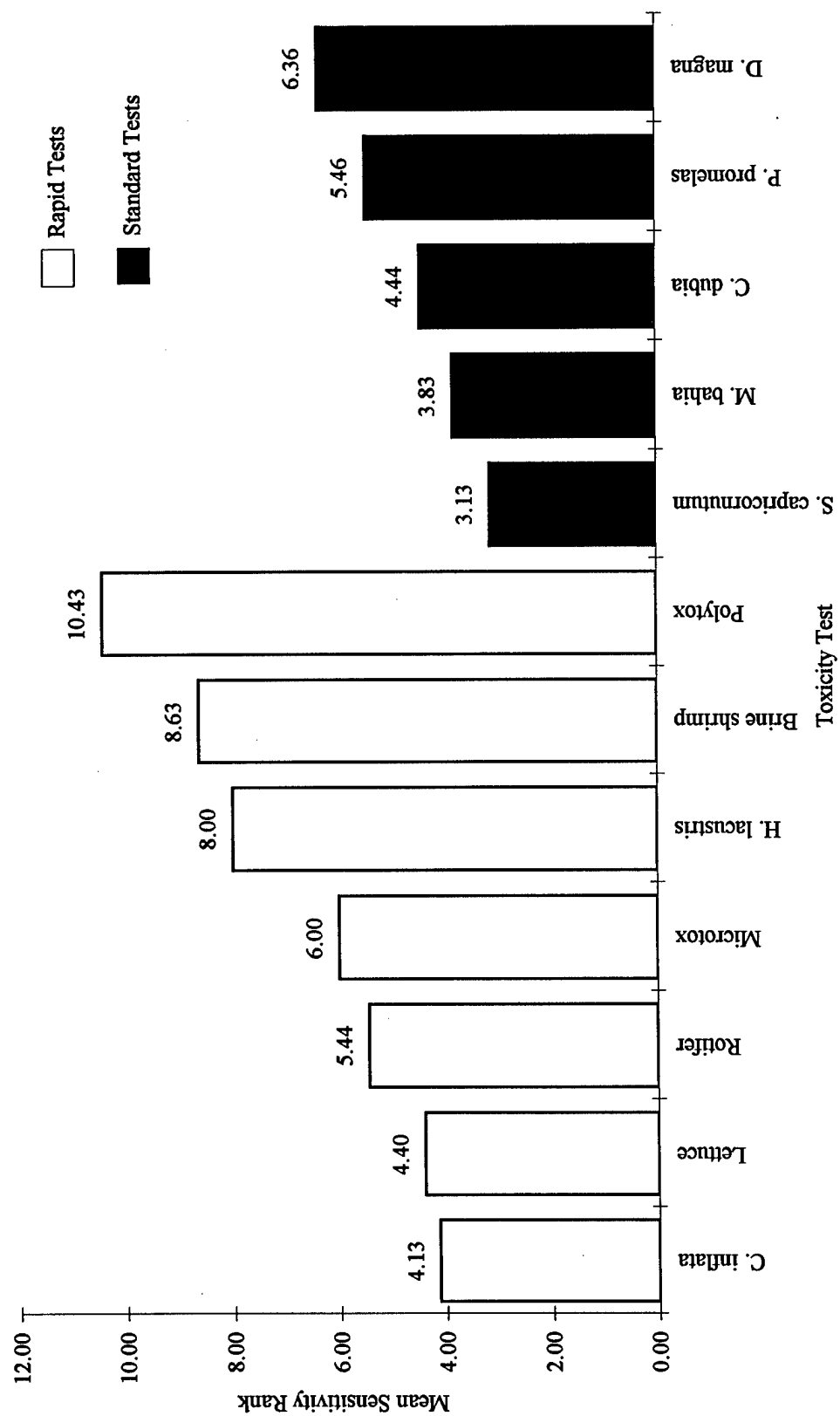


Figure 3.1. Average relative sensitivity rank of rapid-screening and standard acute toxicity tests. Ranks were determined by LC50/EC50 values in Table 3.3. The most sensitive test for each compound was scored as a 1, ranks increased as tolerance increased.

### *Bioavailability*

The toxicity of several compounds appear to be altered by the type of medium used (Table 3.3).

Metals. Cadmium and copper displayed the largest effect between the organic and inorganic media. This difference in sensitivity is most apparent in the dose-response curves (Figures 3.2 and 3.3), which show an obvious difference between all treatments in the organic and inorganic media. For both cadmium and copper, the IG50 value for *C. inflata* dosed in 10% Sonneborn's were significantly different than those dosed in 5% Sonneborn's and minimal salts media. Minimal salts media resulted in the lowest IG50 values and organisms exposed in 5% Sonneborn's displayed IG50 levels between minimal salts and 10% Sonneborn's.

The effects of the organic media on zinc toxicity, were not as apparent. For zinc, ciliates tested in 5% Sonneborn's had the lowest IG50, followed by minimal salts and 10% Sonneborn's media. The IG50 values of these three media were not statistically different. The dose-response of zinc (Figure 3.3) shows an overlap among different media.

Table 3.4. Effects of six compounds on *C. inflata* growth in organic (Sonneborn's) and inorganic (minimal salts) media. Values are IG50s in mg/L. Numbers in parentheses are 95% confidence intervals. A star (\*) indicates a significant difference in response among media. Dashes (--) indicate tests not done.

Compound	Minimal Salts	10% Sonneborn's	5% Sonneborn's
<b>Metals</b>			
Cd	0.020 (0.01-0.032)	0.083* (0.05-0.131)	0.023 (0.02-0.032)
Cu	0.059 (0.02-0.14)	0.58* (1.3-2.3)	0.16 (0.12-0.20)
Zn	0.13 (0.08-0.21)	0.17 (0.10-0.27)	0.080 --
<b>Non-metals</b>			
Ammonia	0.019 (0.013-0.029)	0.017 (0.018-0.027)	--
PCP	0.27 (0.18-0.40)	> 1.9*	--
SDS	52 (38-74)	71 (49-102)	--

Many researchers have demonstrated the occurrence of chelation of metals in organic media, implying that a significant difference in sensitivity could occur due to the bioavailability of the compound (e.g. Dive et al., 1982; Dallinger and Rainbow, 1993). This is an important factor in toxicity testing, and test designs should incorporate the possibility of chelation to test compounds.

Metals have been documented to display the highest change in bioavailability in the presence of organic material (Depledge et al., 1994). The tests conducted in this research show that *C. inflata* was able to respond accordingly to a change in toxicity due to bioavailability. Toxicity of cadmium and copper were found to be significantly different between the 10% Sonneborn's and 5% Sonneborn's and minimal salts. The DOC concentration was measured to be 40 mg/L in 10% Sonneborn's medium and assumed to be close to zero in the minimal salts medium. IG50s were related to the amount of DOC in test media (i.e., high IG50, high DOC concentration). Many studies have demonstrated the decreasing bioavailability of certain substances as the concentration of DOC increases (Dallinger and Rainbow, 1993; Depledge et al. 1994). Almost all of these studies have been conducted on multicellular organisms such as insects, crustaceans, and fish. Research on bioavailability measured by protozoa is scarce. A bioassay investigating the complexation of cadmium with humic substances found that the growth of the ciliate, *C. campylum*, in the presense of Cd responded to changes in humic concentrations (Dive et al., 1982). Results from *C. inflata* in this study, cadmium and copper showed a significant difference between the organic

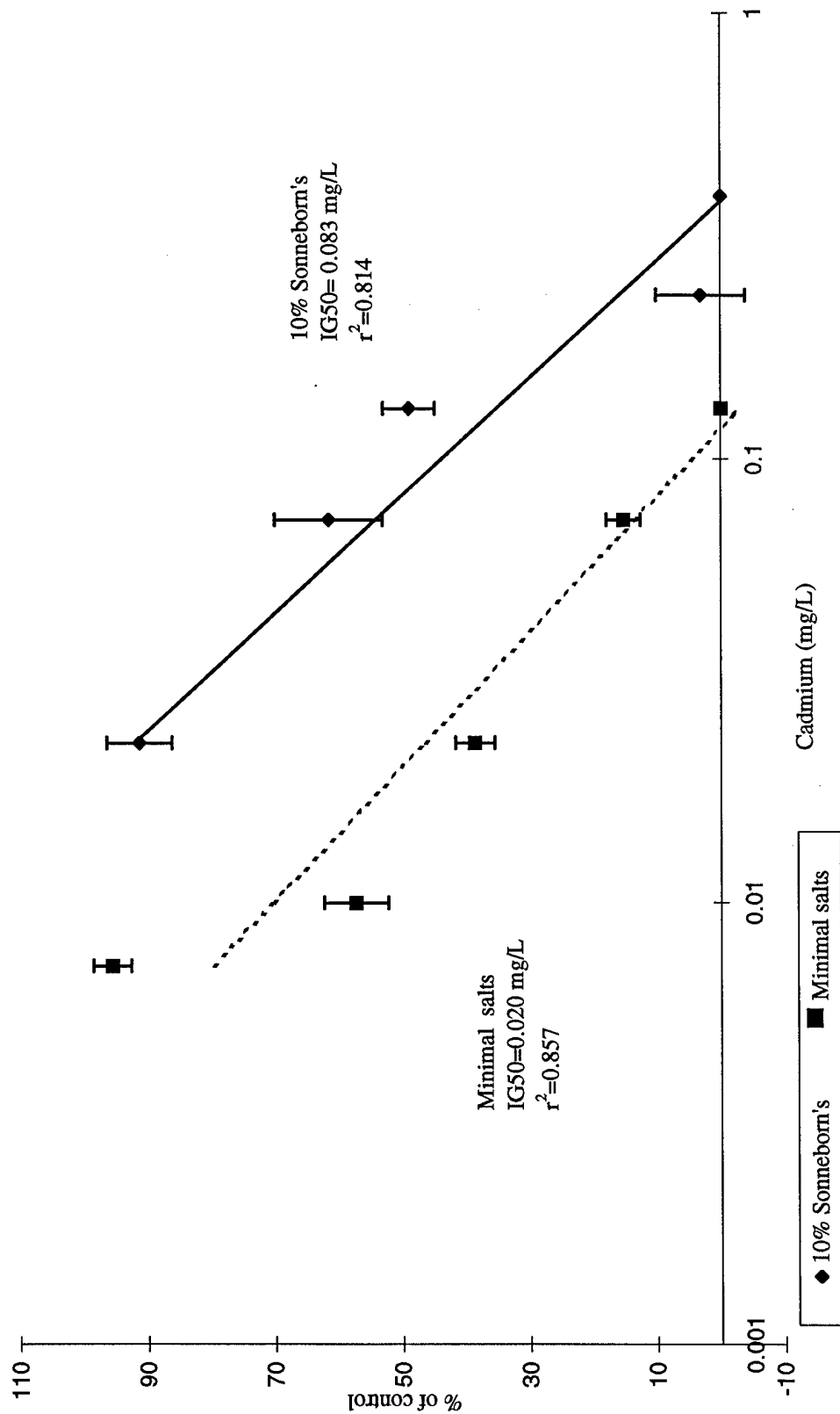


Figure 3.2. Dose-response curve illustrating the effects of cadmium on growth of *C. inflata* tested in organic and inorganic media. Solid line represents 10% Sonneborn's medium, dashed line is response in minimal salts medium.

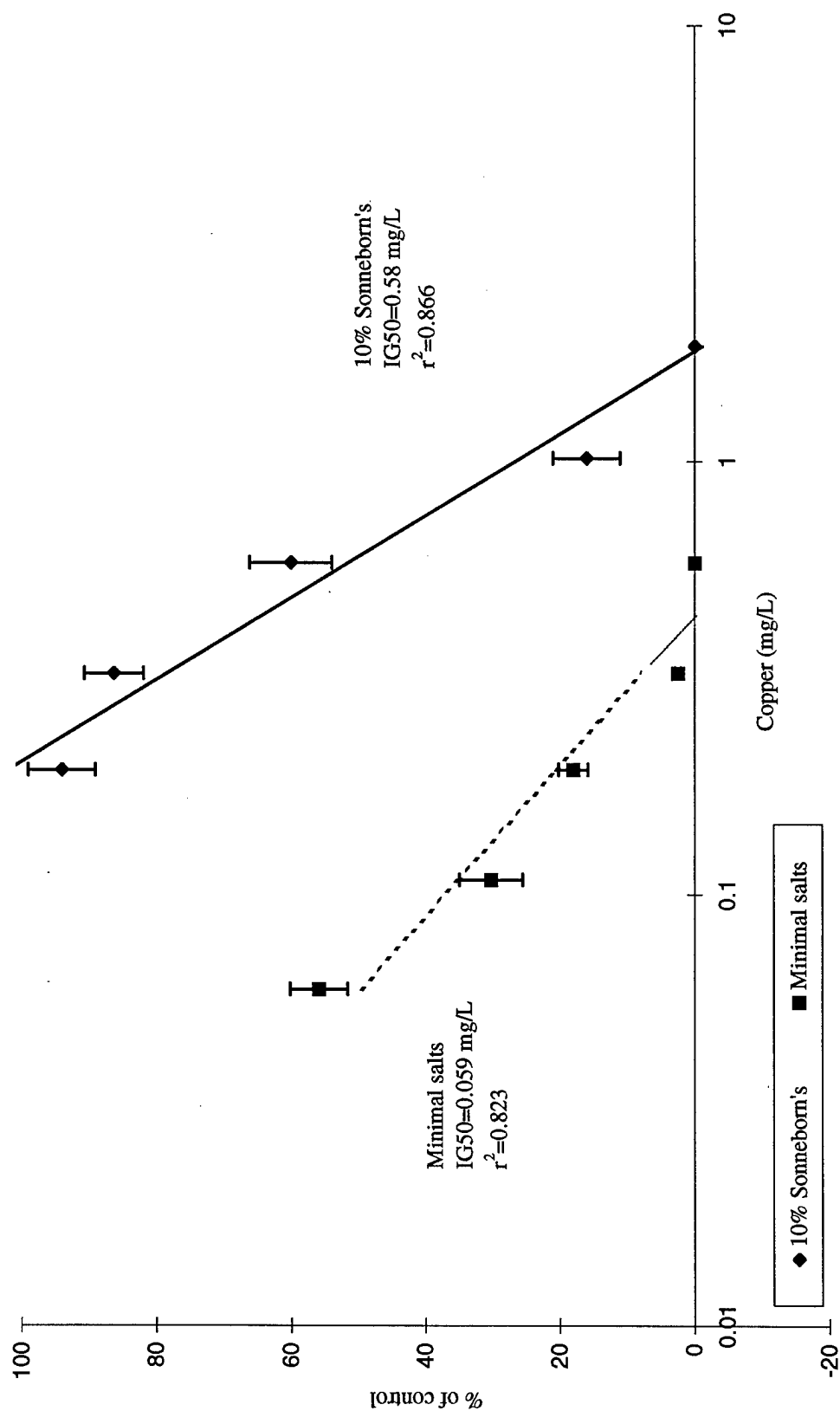


Figure 3.3. Dose-response illustrating the effects of copper on the growth of *C. inflata* tested in organic and inorganic media. Solid line represents 10% Sonneborn's medium, dashed line is response in minimal salts medium.



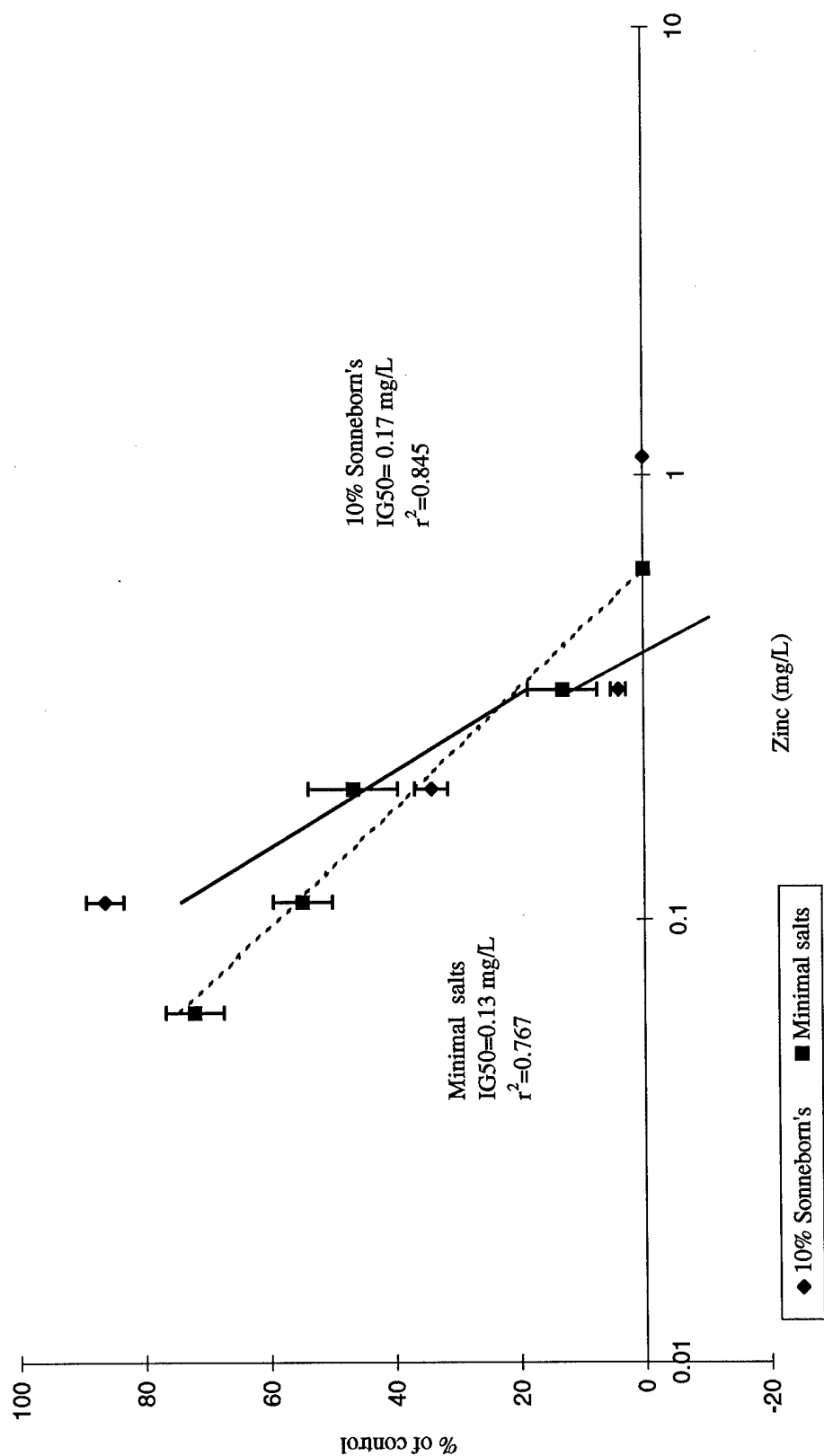


Figure 3.4. Dose-response curve illustrating the effects of zinc on growth of *C. inflata* tested in organic and inorganic media. Solid line represents 10% Sonneborn's medium, dashed line is response in minimal salts medium.

and inorganic media. Thus, much like other bioavailability studies, the rapid-screening test with *C. inflata* is sensitive enough to distinguish complexation of metals with DOC.

Non-metals. Influences of different medium were also apparent in three non-metal compounds. The largest difference in response appeared between the ciliates in the two media dosed with PCP. In the minimal salts media, the IG50 was 0.27 mg/L and in the organic media, ciliate growth was not inhibited at the highest possible dose (1.9 mg/L). In the SDS test, the minimal salts IG50 (52 mg/L) was lower than the IG50 from the 10% Sonneborn's media (71 mg/L), but not significantly different. However, the dose-response curve (Figure 3.5) showed the two responses overlapping in several places (but not at 50% of the control). Ammonia results were opposite of trends shown in other compounds in which the inorganic medium lead to higher toxicity than organic medium. In these tests, ciliates in minimal salts were less sensitive than those in the 10% Sonneborn's media (IG50s were 0.019 mg/L and 0.017 mg/L, respectively). The dose-response (Figure 3.6) showed a slight difference between responses at the higher treatments, although the IG50s were not significant.

There is limited information on the concentration response of aquatic humic substances with regards to organic pollutants. However, several studies have investigated the possibility of PCP binding to natural dissolved organic matter (NDOM). Kukkonen and Oikari (1987) observed that accumulation of labeled PCP by *D. magna* was not affected by humic water (DOC= 23.5 mg/L), even after a 10 hour

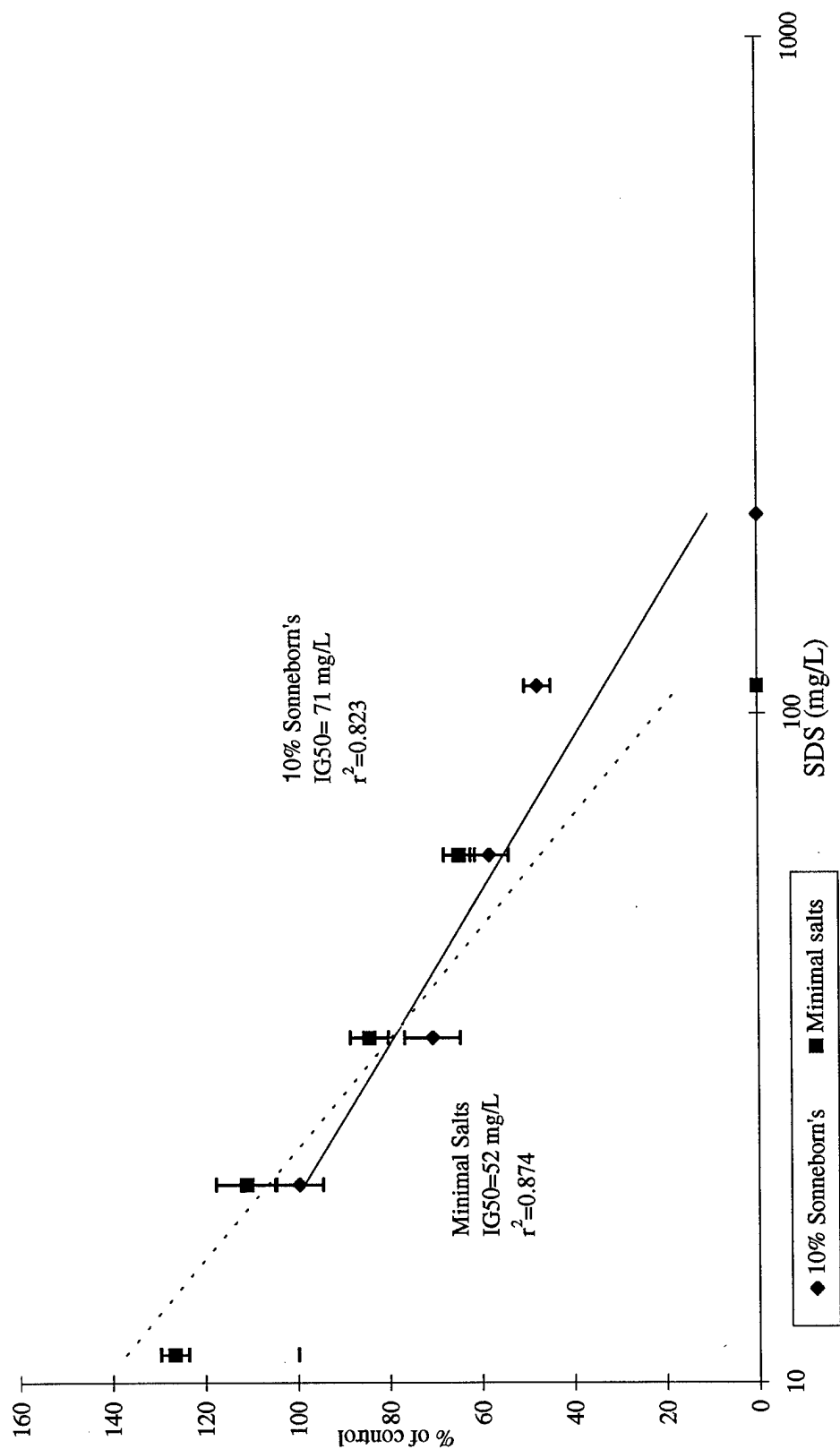


Figure 3.5 . Dose-response illustrating the growth of *C. inflata* to SDS in organic and inorganic media. Solid line represents 10% Sonneborn's medium, dashed line is response in minimal salts medium.

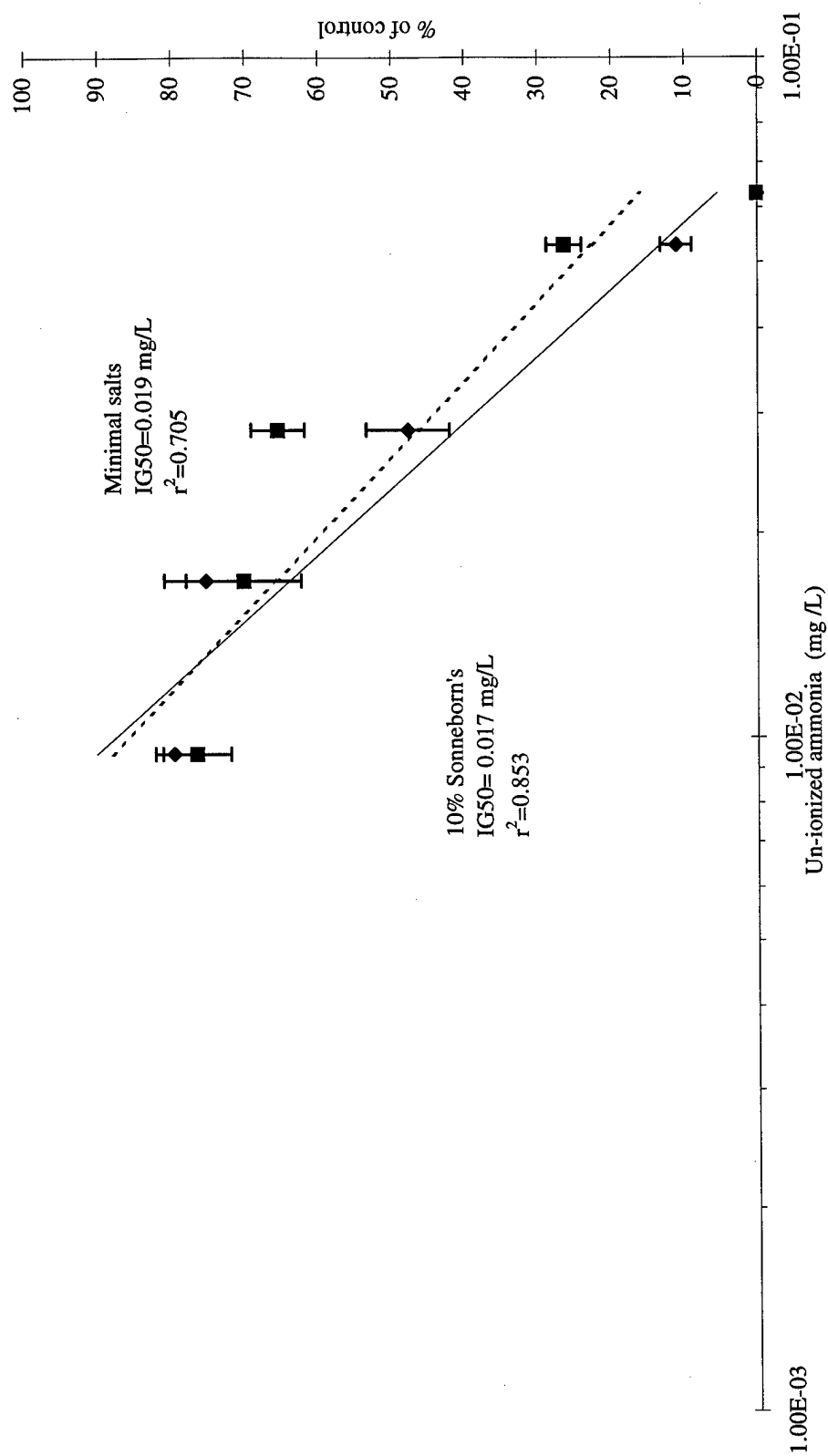


Figure 3.6. Dose-response illustrating effects of un-ionized ammonia on growth inhibition of *C. inflata* tested in organic and inorganic media. Solid line represent 10% Sonneborn's medium, dashed line is response in minimal salts medium.

period. Lee et al. (1993) found that the toxicity of PCP to *D. magna* and the zebrafish, *Brachydanio rerio*, were not altered in the presence of dissolved humic materials (where total organic carbon concentrations were 0-50 mg/L). However, research has shown that PCP does bind to sediments and suspended particles in water (Murray et al., 1981). An important factor of PCP absorption to NDOM (at 15 mg/L as DOC) is pH and that absorption also depends on the concentration of PCP (Lafrance et al., 1994).

Since many studies have shown that the concentration of humic material/ NDOM does not seem to affect bioavailability, there may be some other reason for the difference observed in this research. PCP is known to be highly photodegradable and biodegradable in the soil with a half-life ranging from 15 to 48 days (in anaerobic and aerobic laboratory conditions, respectively). In water, PCP has a half-life ranging from hours to days (Verschueren, 1983). However, stocks of PCP were made in sterilized media the day prior to the testing and refrigerated in amber glass bottles (thus decreasing the possibility of photodegradation). It is possible that the PCP stock made with 10% Sonneborn's was contaminated. Also, the cultures of *C. inflata* certainly contained some bacteria not intentionally added. However, bacteria were also added to the tests with the minimal salts medium so it is unlikely that microbial degradation can be completely responsible for the differences in toxicity.

Slight differences in sensitivity between media, if any, appeared with SDS and ammonia. The growth curve of SDS crosses in several places suggesting that there

really was no effect between media. For ammonia, however, there seemed to be an increasingly large difference in sensitivities as the concentration of ammonia increased. Ammonia can be absorbed on soil and sediment particles (NRC, 1979). However, the results presented here suggest that bioavailability due to complexation with DOC did not occur in this research. Because *C. inflata* appears to be more sensitive in the 10% Sonneborn's than the minimal salts media, it cannot be binding to the high amount of DOC found in 10% Sonneborn's. It is well documented that pH and temperature are crucial factors in determination of ammonia toxicity and ammonia is generally more toxic at relatively high, rather than relatively low, pH (USEPA, 1986). This may be due to the ability of ammonia to cross membranes more readily and cause toxicity (NRC, 1979). Because both these tests were incubated at a constant temperature of 25°C, it is unlikely that temperature played a role in the small difference in sensitivity between these two media. The pH of 10% Sonneborn's was 6.8 and the pH of minimal salts was 5.4, so it is possible that pH may cause this small contrast. While these differences should not be considered significant in this research, future research should investigate this possibility.

### *Pesticides*

Three pesticides were tested: the herbicide 2,4-D, the insecticide malathion, and the fungicide/multipurpose pesticide PCP (Table 3.1). While PCP did not affect *C. inflata* in 10% Sonneborn's (IG50 >1.9 mg/L), in minimal salts media the IG50

was 0.27 mg/L. *C. inflata* population growth was not significantly inhibited in either 2,4-D or malathion in either media at the highest concentrations tested (230 and 99 mg/L, respectively).

Most protozoan pesticide studies have investigated the effects of pesticides on protozoa communities (e.g., change in community structure, respiration, biomass, etc.; see Foissner, 1994). Only a few studies have investigated direct effects on individual species, and even less investigation has been conducted on the pesticides used in this research. Dive et. al. (1980) investigated the effects of several pesticides on the ciliated protozoan, *C. campylum*. Their research found that even at a concentration of 10 ppm, many pesticides were not toxic to this species. Among the most toxic, however, was PCP at a minimal active dose (MAD) of 0.6 mg/L. Both 2,4-D and malathion were not toxic at the highest level tested (10mg/L). Another study by Le Du et al. (1993), also using *C. campylum* as the test species, found that PCP at a concentration of 1.60 mg/L inhibited the growth of 50% of the population. Also compared in this research was the effects of PCP on *Tetrahymena pyriformis*, a freshwater ciliated protozoan. With *T. pyriformis*, the EC<sub>50</sub> of PCP was 0.30 mg/L. *T. pyriformis* was also shown not to be affected by 2,4-D at a concentration of 500 mg/L (Roberts and Berk, 1993).

An important aspect of understanding the toxicity of any compound is understanding its mode of toxic action. This often gives an indication why a pesticide may not be toxic to a particular species.

PCP, a chlorinated hydrocarbon, is a multipurpose pesticide that is currently being phased out of use. It was used mainly as a wood preservative and treatment against fungi and wood-boring insects, but has also been used as a herbicide, molluscicide, algicide, and a disinfectant. The mechanism for toxicity is not well documented (Amdur et. al., 1991); however, it is generally accepted that PCP acts as an uncoupler of oxidative phosphorylation (Cockerham and Shane, 1994; De Bruin, 1976). Oxidative phosphorylation occurs in all living organisms to produce ATP using energy created from the redox reactions of the electron transport system (ETS). In mitochondria, uncouplers like PCP destroy the proton gradient, making the lipid membrane lose hydrogen ions. Electrons can still be passed down the ETS, but because there is no proton gradient, no ATP can be made. ATP is necessary for energy to drive cellular reactions, without the ability to produce ATP, the organism dies (Becker and Deamer, 1991).

Used as an herbicide for selective control of broadleaf plants, 2,4-D (a chlorophenoxy herbicide) stimulates plant growth by mimicking the effect of auxin, a natural growth hormone. As 2,4-D is translocated into all cells, cell proliferation and elongation becomes uncontrolled and disrupts normal plant growth. This leads to a reduction in water uptake, leaf expansion and increased chloroplast production, followed by a softening of the root cortex and overall necrosis (Amdur et. al. 1991). The presence of auxin has not been documented in protozoa. Because *C. inflata* does not contain chloroplasts or plant hormones it is unlikely that it would be toxic by this



mode of action. Many other non-plant test organisms, such as *C. dubia*, *P. promelas*, or brine shrimp (see Table 3.3), also are not very sensitive to 2,4-D.

Malathion, an organophosphorus insecticide, is a neurotoxin that inhibits the enzyme cholinesterase which normally breaks down the neurotransmitter acetylcholine at the synapse. Malathion combines with the active site of cholinesterase, making it unable to work effectively. This results in an accumulation of acetylcholine at the nerve endings, allowing continual neural stimulation and ultimately leading to death of the organism (Becker and Deamer, 1991). Cholinesterase has not been documented in protozoa. *C. inflata* does not have a central nervous system (Meglitsch and Schram, 1991; Foissner, 1993), so malathion is unlikely to significantly affect *C. inflata*. Several metazoan test species are sensitive to malathion, with arthropods such as *D. magna*, being the most sensitive (Table 4).

While 2,4-D and malathion did not negatively affect *C. inflata* growth, there is a possibility that these ciliates may accumulate these toxins. Toxins could become concentrated in organelles, such as digestive vacuoles, or in membranes (Dive et al., 1980; Cooley et al., 1973). While this may seem harmless, it could lead to bioaccumulation. A study on bacteria by Wolfaardt et al. (1994) showed that cell capsules and certain regions of the expolymer matrix can accumulate parent compounds as well as the secondary products. Products also accumulated in biofilms. When protozoa indiscriminately graze on these bacteria and related biofilms, protozoa are also ingesting the accumulated compounds. Several organic compounds, including

PCP were found to have a high bioaccumulation potential to a green alga (Geyer et al., 1981). Research tracing bioaccumulation from protozoa to other animals is rare; however, there is also no strong evidence indicating that bioaccumulation does not occur.

In summary, *C. inflata* did not display a significant difference in growth for 2,4-D and malathion in both media, and PCP did not affect growth in 10% Sonneborn's. Although not investigated in this study, bioaccumulation could possibly occur leading to an effect on other organisms in an ecosystem. *C. inflata* was sensitive to PCP in the inorganic medium, so tests using organic media cannot be completely disregarded in evaluating pesticides.

### 3.2 Supplemental Studies

Field testing. Stream sites 1 and 2 caused no significant effect at the highest level tested (85%). The pH-adjusted groundwater test also showed no significant difference between the controls and the highest dose (87%). However, the unamended groundwater (pH = 4.1) affected protozoan growth. Two groundwater tests were conducted successively, and toxicity results are reported as the percent of groundwater by volume. The first test yielded an IG50 of 37% (23-61%); the NOEC was 18% and the LOEC was 30%. A second test, conducted 24 hr. later, yielded an IG50 of 23% (17-29%); the LOEC was 10% and the NOEC could not be determined. The 95% confidence intervals overlap for the two tests, so the results can not be considered

significantly different. These results suggest that the *C. inflata* growth test is a useful and repeatable field test.

Ingestion rates. Experiments dosed with copper yielded an EC50 of 0.082 mg/L (0.020-0.38 mg/L). This is less than the growth test IG50 using 10% and 5% Sonneborn's, but higher than the minimal salts result (Table 3.4). However, it is only significantly different from the 10% Sonneborn growth test. These results suggest that measuring ingestion rate is comparable to measuring population growth of *C. inflata* in minimal salts medium. The dose-response relationship is shown in Figure 3.7. The EC50 value for feeding (filtration) correspond well to other studies examining the effects of copper on filtration in zooplankton species. For example, Ferrando et al. (1993) reported the copper EC50 for filtration in *Brachionus calyciflorus* as 0.032 mg/L while Flickinger et al. (1982) reported 93% inhibition of filtration rate at copper concentrations of 0.01-0.04 mg/L.

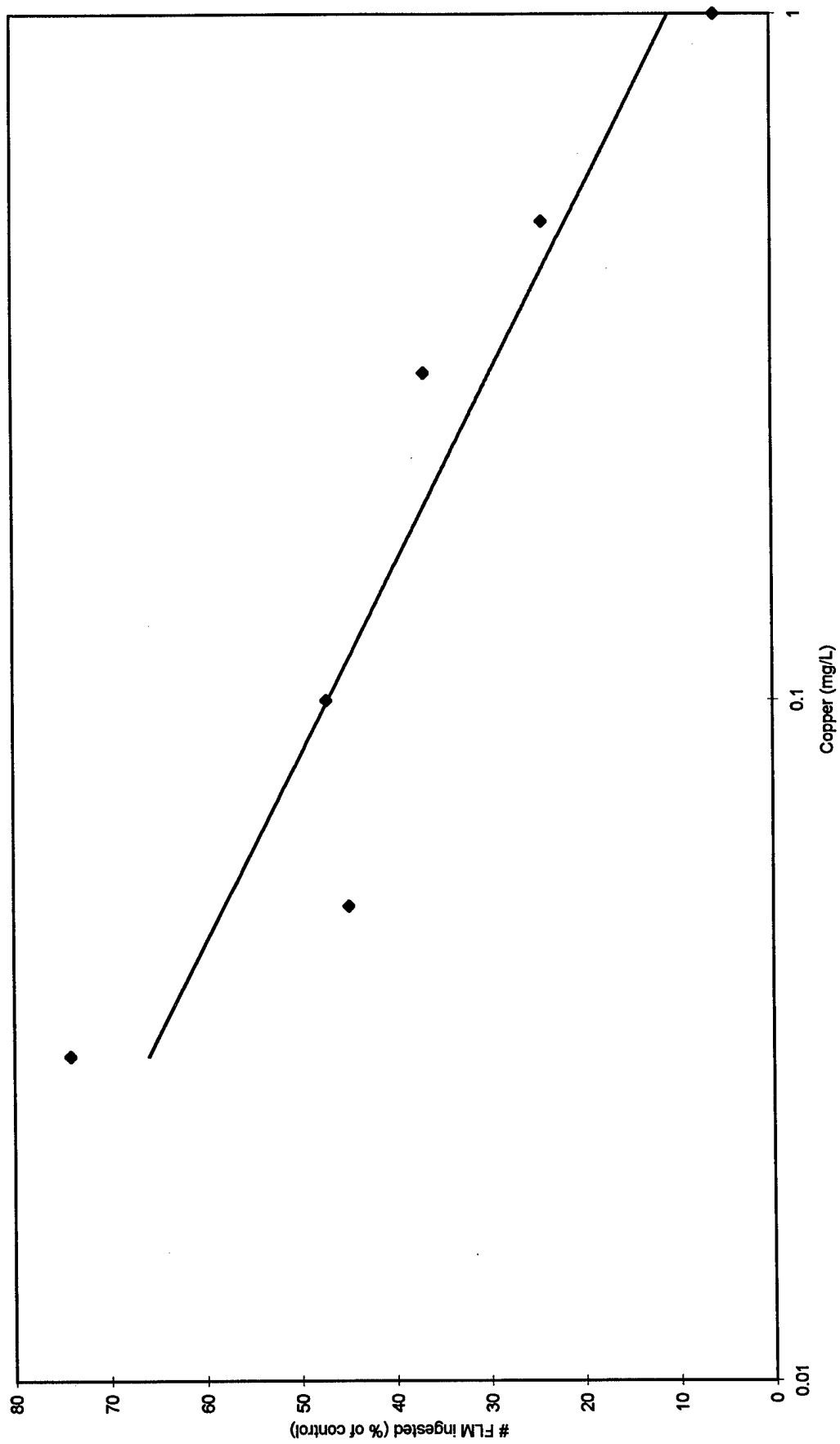


Figure 3.7. Dose-response curve showing the effects of copper on ingestion rates of *C. inflata*.

### 3.3 *Haematococcus lacustris*

*H. lacustris* displayed varying sensitivity to the seven compounds tested (Table 3.5). The rank order of sensitivity was: Cu > PCP > Cd > Zn > SDS > octanol > 2,4-D. *H. lacustris* was found to be sensitive to the three metals tested. Also toxic was PCP, a multi-purpose biocide, acting as a cell uncoupler of oxidative phosphorylation. SDS is a surfactant and may have disrupted the cell membrane. 2,4-D is a chlorophenoxy compound used as an herbicide against broad-leafed plants. It is a growth stimulant, mimicking the plant hormone auxin and causing uncontrolled growth. Since *H. lacustris* is not a broad-leafed plant controlled by auxin the effects of this herbicide is minimal.

A comparison in sensitivity between standard test organisms, rapid- bioassay tests and *H. lacustris* is shown in Table 3.3 and Figure 3.1. When ranked with eleven other bioassays, *H. lacustris* was the third most tolerant, followed by the brine shrimp and Polytox. Compared with the test alga, *Selenastrum capricornutum*, *S. capricornutum* was more sensitive to most compounds tested than *H. lacustris*. However, *H. lacustris* was more sensitive to 2,4-D and was found to be closely related in sensitivity to copper and PCP. *S. capricornutum* has a similar a rank order of sensitivity : Cd; Cu > Zn > PCP > SDS > 2,4-D to *H. lacustris*.

Due to the seemingly tolerant nature of *H. lacustris*, and the inconvenience of manually enumerating cells, its use as a test organism is not as practical as *C. inflata*. However, it may be possible that a change in methodology would increase sensitivity

as well as ease of operation. Instead of manually counting individual cells (which could also be done by modifying hemocytometer methods), it would be possible to estimate growth with an automatic counter or through fluorometry (by measuring chlorophyll a).

Table 3.5. Sensitivity (expressed as 50% inhibition of growth of the population [IG50], no observed effect concentration[NOEC], and lowest observed effect concentration [LOEC]) of *H. lacustris* to seven compounds in 24 hr tests. Values in parentheses are 95% confidence intervals from inverse prediction. All values in mg/L. Dashes (--) indicate values that could not be determined.

Compound	IG50	LOEC	NOEC
Cadmium	0.68 (0.47-0.97)	0.56	0.18
Copper	0.093 (0.63-0.14)	0.056	--
Zinc	5.1 (2.4-8.8)	5.6	3
2,4-D	70 (37-183)	100	56
Octanol	54 (35-92)	56	30
PCP	0.67 (0.45-1.0)	1	0.56
SDS	44 (34-58)	30	18

## 4.0

## CONCLUSIONS

Evaluation of the rapid-screening toxicity test using *C. inflata*, has yielded several conclusions. First, this test is sensitive enough to respond to a variety of compounds. Ranges of IG50s are comparable to other protozoan bioassays as well as other rapid-screening and standard tests. *C. inflata* was not the most sensitive to all compounds tests, however it was intolerant to many. In fact, when ranked with other tests, *C. inflata* was found to more sensitive than standardized tests using organisms such as *D. magna*, *C. dubia*, and *P. promelas*. *C. inflata* also ranked higher in sensitivity than many of the rapid-screening tests currently used (like Microtox®, Polytox®, rotifer, and the brine shrimp).

This test is sensitive enough to respond to differences in toxicity due to bioavailability. Tested in both organic and inorganic media, *C. inflata* displayed a higher tolerance to compounds tested in the organic media. This was especially true for cadmium and copper. This test is sensitive enough to detect chelation of metals by DOC.

Lastly, as in other protozoa and several other organisms, there was no significant effect found for two of the three pesticides tested. This may be simply because the physiological characteristics of protozoan and the pesticides' modes of action do not allow certain responses to be measured. Because *C. inflata* was affected

by one pesticide, it would be unwise to overlook using protozoa bioassays for pesticide testing.

Supplemental studies showed that the ciliate model and test can be used in field testing and that responses other than growth (i.e., feeding or filtration rate) may be useful for reducing the test period while still measuring an ecologically and toxicologically sensitive and meaningful response. Since the test medium was a minimal salts medium resembling low ionic strength waters, it should be possible to adjust the test medium to general chemistry of putatively contaminated surface or ground water. That is, shifting the test medium to reconstituted water of defined hardness should be possible.

*C. inflata* is an easily cultured protozoan that occurs world-wide. With this test, effects of compounds can be assessed quickly and inexpensively. Responses are similar to those of other test organisms, and results are repeatable. This test would be a worthy addition to use in a battery-of-tests approach.

The utility of *H. lacustris* in a battery of rapid toxicity tests is somewhat more problematic. Its sensitivity is not as great as other "plant" models, even though a few other rapid tests are generally less sensitive. Additionally, direct cell counting is more difficult than in the ciliate tests. On the other hand, *H. lacustris* can be stored in the cyst form and cultured when needed. Preliminary work has shown that other, wild-type strains may have better sensitivity and more rapid growth rates than the ATCC strain of *H. lacustris*, and additional research may be needed to identify and develop



these additional strains. A small number of other species of *Haematococcus* are known, and these species may display greater toxicant sensitivity while retaining the ability to produce resistant cysts. Further, other methods to assess algal growth (automated cell counting, fluorometry) could simplify endpoint assessment in this test.

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## APPENDIX A

In addition to the final test methods previously listed, several preliminary experiments were performed which were essential to the success of the toxicity tests. This section describes those experiments.

*Growth*

The objectives of these experiments were two-fold: 1) to determine the minimal dilution of Sonneborn's medium that would supply enough nutrients for adequate growth and 2) determine average growth after a 24 and 48 hour test periods.

Tests were set up following the procedures for the toxicity test, except no toxicants were added (so all wells were "controls") and each test well contained a total of 1 ml. The three dilutions of Sonneborn's media tested were 10%, 5% and 1%. All media were autoclaved prior to use. After 24 hours, the wells were counted and cell number recorded. Cells were then refreshed with 100  $\mu$ l of media (10%, 5%, or 1%) and counted again 24 hours later (48 hour total time). This experiment was repeated several times.

After both 24 and 48 hours, growth in the 10% medium was the highest, followed by 5%. Little to no growth occurred in 1% Sonneborn's. In a 24 hour period, growth averages in 10% Sonneborn's medium ranged from 597 cells ( $\pm$  29.9) - 1474 cells ( $\pm$  285), 5% ranged from 288 cells ( $\pm$  67) - 637 cells ( $\pm$  141), 1% averages extended from 0 - 125 cells ( $\pm$  38). Counting the next day generally revealed higher cell numbers. One minor test indicated that 3% did not yield adequate growth. From



this, it was determined that 10% and 5% Sonneborn's dilutions generally yielded a high enough cell count to perform toxicity testing.

#### *Variability*

Because the 24 well plates contain wells which are in different "positions" (i.e., middle of the plate, edges) and the toxicity test would most easily be set up in a non-random fashion (to avoid confusion of which test wells were which), preliminary tests were conducted to ascertain variability between wells in a single plate.

One full tissue culture plate test (24 wells) was performed with 10% Sonneborn's. Other tests were conducted using half of a plate (12 wells) with 10% and 5% Sonneborn's. All media was autoclaved before use and total media and culture volume always equaled 1 ml. The test period was 24 hours. An ANOVA procedure was performed on the data obtained to determine if significant differences occurred between wells.

Growths for all tests were high over a 24 hour period. ANOVA results showed no significant differences between wells of any of the plates. Growth means and standard deviations for the 12 well test were: 10%,  $1047 \pm 270$  cells; 5%  $910 \pm 61$  cells. In the 24 well plate growth and standard deviations were  $860 \text{ cells} \pm 122$ .

#### *Bacteria*

The use of the food source *Klebsiella pneumoniae* was necessary for the tests conducted in the inorganic minimal salts media. Experimentation with methods relating to bacteria were investigated before a standard method was found.

When tests first began, bacteria were grown in flasks of 10% Sonneborn's media. Growth was slow and it was found that adding a few drops of peptone increased growth rates rapidly. Optical densities were not taken; growth increase was simply determined on the visual turbidity of the culture. If the culture was cloudy, 100 ml (divided into two 50 ml tubes) was spun in the ultracentrifuge at approximately 13,000 RPMs for 20-30 minutes. Then the liquid was decanted and the pellet was re-suspended in 10-15 ml sterile minimal salts media. Tubes were covered with parafilm wax and vortexed until adequately mixed. This bacterial mixture was then distributed as media and the food.

There are many problems with this method. First, since the exact amount of this slurry was distributed based on the calculations of toxicant, media, and cell volume, this quantity, and thus the amount of bacteria, varied per concentration (i.e., less bacteria would be in high concentrations where more compound stock was added). Second, the bacteria were centrifuged from liquid media which may have contained other organisms. The number of bacteria varied from test to test. Lastly, this method would be impractical for use in the field and also requires constant bacterial culturing. The method of growing the bacteria on agar plates, scraping, and freezing (described in the methods section) is much more conducive to the principles of rapid-screening toxicity testing.

In order to further standardize the addition of *K. pneumoniae*, investigations were done to determine the approximate number of bacteria needed to sustain *C.*

*inflata* for 24 hours. Because no more than 200  $\mu$ l of *C. inflata* cells were ever added to each well, a fixed volume of 200  $\mu$ l bacteria would also be added to each well. A test was set up using 200  $\mu$ l of varying optical densities (OD) of bacteria in each well. Bacteria were prepared in the same manner described in the methods, and after optical density was read, 1 ml of the bacteria sample was fixed (10:1) with 10% buffered glutaraldehyde (GTA). Minimal salts media, ciliates and 200  $\mu$ l of bacteria were added to two wells. The slurry was then diluted and the process was repeated. Twenty-four hours later the wells were inspected for growth. It was determined that the lowest OD for adequate growth was approximately 0.2 (400 nm).

The fixed samples of *K. pneumoniae* at this optical density were counted by epifluorescence staining. The samples were filtered on sterilized equipment, stained with 4',6-diamidino-2-phenylindole (DAPI). After filtration, filters were placed on a cleaned slide and air dried. Counting was done under fluorescence at 1000X.

Organisms were enumerated by counting all bacteria occurring in randomly selected grids of a standard Whipple Grid. A minimum of 10 grids were counted per slide.

Calculations determined the average number of cells per ml to be  $3.2 \times 10^7$ . Thus the 200  $\mu$ l food source added to each well in these toxicity tests contained approximately  $6.8 \times 10^6$  *K. pneumoniae*.

## APPENDIX B

This is a description of the chemical methods used by Coffey Laboratories, Inc. to analyze stock concentrations.

## CFR 200.7 - cadmium, copper, sodium, zinc

This method is used to determine concentration of metals and trace elements by inductively coupled plasma-atomic emission spectrometry. Measurement of atomic emission was done by an optical spectrometric technique. This analysis was used for cadmium, copper, sodium (for SDS) and zinc (USEPA 1994). Currently there is no good way to directly measure SDS concentration. Hence, sodium is measured by this method and SDS is then calculated from the molecular formula of SDS (Harvey Jackson, Coffey Laboratories, pers. comm).

## CFR 604 - PCP, phenol

This method involves flame ionization detector gas chromatography (FIDGC) and can be used in the determination of PCP. A gas chromatograph/mass spectrophotometer (GC/MS) is used for confirmation of the results (USEPA, 1994).

## EPA 614 - malathion

This is a gas chromatographic (GC) method used to detect organophosphorous pesticides such as malathion. A measured volume of sample is solvent extracted w/

15% methane chloride in hexane. The extract is then dried and concentrated. GC is performed by flame photometric (FP) or thermionic bead gas chromatography (Pressley and Longbottom, 1982a).

#### EPA 615 - 2,4-D

A GC method used to determine 2,4-D in solution. A measured volume of sample is acidified. Acid herbicides and their salts are extracted with ethyl ether. Derivatives are hydrolyzed with potassium hydroxide and the extra organic material is then removed with a solvent wash. The remaining extracted acids are converted to their methyl esters with diazomethane. Excess reagents are removed and esters are determined by electron capture (EC) gas chromatography. (Pressley and Longbottom, 1982b).

#### SM 4500, A-D - ammonia

This is a series of methods used to determine the concentration of ammonia in an aqueous sample. Section A discusses selection of methods, possible interfaces and proper storage of samples (acidification to pH <2, and storage at 4°C). Section B describes a preliminary distillation step, which is necessary to complete prior to performing method C. Preliminary distillation involves buffering the sample at pH 9.5 (to decrease hydrolysis of organic nitrogen compounds) and distillation into a solution of boric acid for the titrimetric method (C) or into a solution of H<sub>2</sub>SO<sub>4</sub> for the

ammonia-selective electrode method (D). Coffey Laboratories used methods C and D to detect ammonia in stock sample. Method C, the titrimetric method, involves titration of ammonia in distillate with a standard titrant until the indicator is a pale lavender color. Concentration of ammonia is then calculated mathematically. The ammonia-selective electrode method uses a hydrophobic gas-permeable membrane to isolate the sample solution from an electrode in an internal solution of ammonium chloride (APHA, 1989). Un-ionized ammonia was calculated and corrected for pH and temperature (USEPA, 1986)